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**J. EXP. MED., April 1988, vol. 167, US; P. SECKINGER et al.: "A human inhibitor of Tumor Necrosis Factor alpha"**

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**Description**

This invention relates to Tumor Necrosis Factor (TNF) Inhibitory Protein, salts, functional derivatives and active fractions thereof, having the ability to inhibit the binding of TNF to its receptors and the cytotoxic effect of TNF and which can be used against the deleterious effects of TNF. The invention also relates to a process for the purification of said TNF Inhibitory Protein and to the substantially purified protein, to its cloning and its production by recombinant DNA techniques. It further relates to pharmaceutical compositions comprising such a protein, or salts, functional derivatives and active fractions thereof, for protecting against the deleterious effects of TNF.

Tumor Necrosis Factor-alpha (TNF-alpha) and Lymphotoxin or TNF-beta (hereinafter, TNF refers to both TNF-alpha and TNF-beta) are cytokines which have many effects on cells (Wallach D. (1986), in: Interferon 7 (Ion Gresser, Ed.), pp.83-122, Academic Press, London, and Beutler B. and Cerami A. (1987), New England J. Med. 316: 379-385). Both TNF-alpha and TNF-beta initiate their effects by binding to specific cell surface receptors. Some of the effects are likely to be beneficial to the organism: they may destroy, for example, tumor cells or virus infected cells and augment antibacterial activities of granulocytes. But, quite clearly, both TNF-alpha and TNF-beta have also effects which can be extensively deleterious. There is evidence that over production of TNF-alpha can play a major pathogenic role in several diseases. Thus effects of TNF-alpha, primarily on the vasculature, are now known to be a major cause for symptoms of septic shock (Tracey K.J. et al. (1986) Science 234: 470-474). In some diseases, TNF may cause excessive loss of weight (cachexia) by suppressing activities of adipocytes and by causing anorexia and TNF-alpha was thus called cachectin. It was also described as a mediator of the damage to tissues in rheumatic diseases (Beutler, op. cit.) and as a major mediator of the damage observed in graft-versus-host reactions.

There is therefore a necessity in finding out ways to eliminate or antagonize endogenously formed or exogenously administered TNF. Our first attempt in this direction was the development of monoclonal antibodies which neutralize the TNF-alpha cytotoxic activity and were shown to protect mice against the lethal effect of TNF-alpha under conditions mimicking elicitation of septic shock (as described in our European Patent Application EP 186 833 published on July 9, 1986). However, therapy with murine monoclonal antibodies, especially if administered repetitively, may not always be advisable in humans. Therefore the need was felt for development of biological agents which could similarly antagonize the deleterious effects of TNF.

Prior to the filing date of the priority application of the present application, there was no information as to the existence of biological agents which could antagonize the cytotoxic activity of TNF. There were publications describing uromodulin, a 85-kDa immunosuppressive glycoprotein isolated from the urine of pregnant women (Muchmore, Andrew V. and Decker, Jean M. (1985) Science 229: 479-481), that was shown to be a high affinity ligand for and a potent inhibitor of interleukin 1 (IL-1) (Muchmore, Andrew V. and Decker, Jean M. (1986) J. Biol. Chem. 261: 13404-13407; Brown, K.M. et al. (1986) Proc. Natl. Acad. Sci. USA 83: 9119-9123). Uromodulin was later shown to be identical to the Tamm-Horsfall glycoprotein, the most abundant protein of renal origin in normal urine (Pennica, Diane et al. (1987) Science 236: 83-88). Another inhibitor of IL-1 found in the urine of febrile patients was disclosed in some publications (Liao, Zenghua et al. (1984) J. Exp. Med. 159: 126-136; Seckinger, Phillippe et al. (1987) J. Immunol. 139: 1546-1549). It was shown that this urine inhibitor of IL-1 affects numerous biological activities of both forms of recombinant IL-1, IL-1 alpha and IL-1 beta to the same extent. Although human TNF-alpha shares some of the biological activities of IL-1, this IL-1 inhibitor did not inhibit the biological activities of TNF-alpha (Seckinger, Phillippe et al. (1987) J. Immunol. 139: 1541-1545).

Subsequent to the filing date of the priority application of the present application, it was disclosed that uromodulin and the Tamm-Horsfall glycoprotein bind recombinant IL-1 alpha, IL-1 beta and TNF-alpha in a lectin-like interaction and it was suggested that it may play an important role in the regulation of circulating levels of these lymphokines (Hession, Catherine et al. (1987) Science 237: 1479-1484). Although uromodulin does not inhibit the cytotoxic activity of TNF-alpha as monitored by lysis of tumor cell targets, it interacts with recombinant TNF-alpha via carbohydrate chains and this interaction may be critical in promoting clearance and/or reducing in vivo toxicity of TNF and other lymphokines (Sherblom, Anne P. (1988) J. Biol. Chem. 263: 5418-5424). In a recent publication by Seckinger et al. (J. Exp. Med. (1988) 167: 1511-1516) a human inhibitor of TNF-alpha obtained from the urine of febrile patients was described as a 40-60 Kda protein inhibiting the cytotoxic activity of TNF-alpha. It was shown to differ from uromodulin and from the above-mentioned IL-1 inhibitor.

The present invention provides TNF Inhibitory Protein, salts, functional derivatives and active fractions thereof, which can antagonize the effects of TNF. This antagonism can be determined both by measuring

reduction of the cytotoxic activity of TNF as well as by measuring interference with TNF binding to its receptors.

The invention is directed also to said TNF Inhibitory Protein in substantially purified form, being free of proteinaceous impurities.

5 The invention also relates to a process for the purification of TNF Inhibitory Protein.

The invention further relates to recombinant DNA molecules comprising the nucleotide sequence coding for said protein, expression vehicles comprising them and host cells transformed therewith and to a process for producing the TNF Inhibitory Protein by culturing said transformant cells in a suitable culture medium.

10 The TNF Inhibitory Protein of the invention and its salts, functional derivatives and active fractions thereof are for use as active ingredients of pharmaceutical compositions to protect mammals against the deleterious effects of TNF.

#### DESCRIPTION OF THE FIGURES

15 Figure 1A shows the elution pattern of the TNF Inhibitory Protein from an Ultrogel ACA 44 gel filtration column. Two (2) ml fractions were collected and tested for protein content by absorption at 258 nm (—), for interference with  $^{125}\text{I}$ -TNF-alpha binding to its cell surface receptor (x-x) and for inhibition of TNF-alpha cytotoxic activity (●-●). The major peak of TNF inhibitory activity eluted slightly before the majority of the protein.

20 Figure 1B shows the elution pattern of the TNF Inhibitory Protein when dialyzed against water prior to application on the Ultrogel ACA 44 gel filtration column. Two ml fractions were collected and assayed as in Fig. 1A. The dialysis against water did not change the elution pattern when compared with Fig. 1A.

Figure 2 shows the morphology of murine A9 cells treated with cycloheximide (CHI) (a), with TNF-alpha-CHI (b) and with TNF-alpha-CHI together with the TNF Inhibitory Protein (c).

25 Figure 3 shows the results of the second step of the purification of the TNF Inhibitory Protein. Carboxymethyl (CM) Sepharose purified TNF Inhibitory Protein was loaded in 8 x 2 ml portions on a Mono S 5/5 cation exchange column and eluted with a linear gradient from 0 to 350 mM NaCl (----) in a buffer containing 10 mM citric acid, 0.02% sodium azide, pH 5.0. At a flow rate of 0.5 ml/minute, fractions of 0.5 ml were collected and assayed for inhibition of TNF cytotoxicity on murine A9 cells. The majority of the 30 TNF Inhibitory Protein eluted at a salt concentration of 180 to 200 mM NaCl

(█████) .

35 The protein was monitored by absorption at 280 nm (—).

Figure 4 shows the results of the third step of the purification of the TNF Inhibitory Protein. The active protein obtained by purification on CM-Sepharose and Mono S was dialyzed against a buffer containing 5 mM sodium borate, 0.02% sodium azide, pH 9.0 and loaded on a Mono Q 5/5 anion exchange column. The bound proteins were eluted at a flow rate of 0.5 ml/minute with a linear salt gradient from 0 to 60 mM NaCl and then from 60 to 300 mM NaCl (----). Fractions of 0.5 ml were collected and tested for inhibition of TNF 40 cytotoxicity on murine A9 cells

(█████) .

45 The protein was monitored during elution by measuring the absorption at 280 nm (—). As shown, most of the activity eluted at a salt concentration of 30 to 40 mM.

Figure 5 shows the separation of TNF Inhibitory Protein on reversed phase HPLC. The active protein eluted from Mono Q 5/5 was injected in one 1.6 ml portion on an Aquapore RP-300 HPLC column (Brownlee Labs) run with 0.3% aqueous TFA (buffer F) in water at a flow rate of 0.5 ml/minute. The column was then eluted with a linear gradient of acetonitrile in buffer F from 0 to 20% for 5 minutes, followed by a linear gradient from 20-50% for 60 minutes and then with a linear gradient from 50-80% for 5 minutes (----). Fractions of 0.5 ml were collected and tested for inhibition of TNF cytotoxicity on murine A9 cells. The protein concentration was monitored during elution by measuring relative fluorescence of representative samples of each fraction, after automated reaction with fluorescamine (—). The TNF inhibitory activity eluted as a sharp peak together with an isolated peak of protein.

Figure 6: Samples of the active material of each step of the purification.

The TNF Inhibitory Protein of the invention may be found in human urine. When crude preparations thereof derived from human urine concentrate were chromatographed on ultrogel ACA 44 gel filtration column, it showed an apparent molecular weight of 40-80 kDa. The substantially purified protein, which is substantially free of proteinaceous impurities, has a molecular weight of about 26-28 Kda when analyzed by 5 SDS PAGE under reducing conditions and it moves as a single peak on reversed-phase high performance liquid chromatography (HPLC). The isoelectric point of the active protein determined by isoelectric focusing is between pH 6 and 8. Its activity is determined by its ability to inhibit the binding of TNF-alpha to its surface receptors on human HeLa and FS11 fibroblast cells and/or by its ability to inhibit the cytotoxic effect of TNF-alpha on murine A9 cells.

10 It is further characterized by containing at the N-terminus the following amino-acid sequence:

1	5	10	15
<b>Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser</b>			

15 wherein the amino acid designated X at the 14th position was not identified and the presence of cysteine (Cys) at the 4th position is theoretical, since PTH (Phenyl thiohydantoin) Cys cannot be identified as such and no other residue was detected in this position.

20 As used herein the term 'salts' refers to both salts of carboxyl groups and to acid addition salts of amino groups of the protein molecule which have the ability to inhibit the binding of TNF to its receptors and to inhibit the cytotoxic effect of TNF on cells. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as 25 triethanolamine, arginine or lysine, piperidine, procaine and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids such as, for example, acetic acid or oxalic acid.

30 "Functional derivatives" as used herein covers derivatives having the ability to inhibit the binding of TNF to its receptors and to inhibit the cytotoxic effect of TNF on cells which may be prepared from the functional groups which occur as side chains on the residues or the N- or C-terminal groups, by means known in the art and are included in the invention as long as they remain pharmaceutically acceptable, i.e. they do not destroy the activity of the protein and do not confer toxic properties on compositions containing it.

35 These derivatives may, for example, include aliphatic esters of the carboxyl groups, amides of the carboxyl groups by reaction with ammonia or with primary or secondary amines, N-acyl derivatives of free amino groups of the amino acid residues formed with acyl moieties (e.g. alkanoyl or carbocyclic aryl groups) or O-acyl derivatives of free hydroxyl group (for example that of seryl or threonyl residues) formed with acyl moieties.

40 As "active fractions" of the TNF Inhibitory Protein, the present invention covers any fragment or precursors of the polypeptide chain of the protein molecule alone or together with associated molecules or residues linked thereto, e.g. sugar or phosphate residues, or aggregates of the protein molecule or the sugar residues by themselves, provided said fraction has the ability to inhibit the binding of TNF to its receptors and to inhibit the cytotoxic effect of TNF on cells.

45 The present invention relates also to mixtures of any of the foregoing.

#### 1. PRELIMINARY CHARACTERIZATION AND INITIAL PURIFICATION OF THE TNF INHIBITORY PROTEIN

In a preliminary characterization in the crude state, the following properties and activities of the protein were observed:

- 50 a) The TNF inhibitory activity could be found in the urine of healthy as well as of sick donors;
- b) The active protein was not dialyzable through membranes with a molecular weight cut-off of 10 kDa;
- c) The apparent molecular weight of the active TNF Inhibitory Protein, when chromatographed on an Ultrogel ACA 44 gel filtration column, was found to be between 40 and 80 kDa. Extensive dialysis against water did not change the behaviour of the protein in this procedure (Fig. 1A and 1B);
- 55 d) The isoelectric point of the active protein as determined by preparative isoelectric focusing was between pH 6 and 8,
- e) The active protein bound in part to Concanavalin-A Sepharose and could be specifically eluted with methyl-alpha-D-mannopyranoside which suggests that the protein is glycosylated;

- f) The TNF inhibitory activity was heat labile;
- g) A variety of protease inhibitors did not interfere with the biological activity of the TNF Inhibitory Protein indicating that the mechanism underlying the TNF inhibition could not be explained by proteolytic activities present in crude urine; and

5 h) Inhibition of the binding of TNF-alpha to its cells surface receptors occurred only when the crude protein mixture containing the TNF Inhibitory Protein was applied simultaneously with TNF (Table 1).

The TNF Inhibitory Protein of the present invention differs thus from uromodulin by several of the above characteristics, such as by (a) its apparent molecular weight in gel filtration, (b) its isoelectric point, and (c) the fact that no extensive aggregation of the protein could be observed when dialyzed against water.

10 Partially purified TNF Inhibitory Protein preparations were obtained by fractionation of the urinary proteins by gel filtration, according to the following procedure: Urine was concentrated by ultrafiltration with a membrane of a molecular weight cut-off of 10 kDa and then further by ultrafiltration with a membrane of a molecular weight cut-off of 5000 (Amicon YM5 membrane). The concentrate was dialyzed against PBS (Phosphate buffered saline) containing 1 mM Mg<sup>2+</sup>, 1 mM Ca<sup>2+</sup> and then loaded on a Concanavalin-A  
15 Sepharose column equilibrated with the same buffer. The column was washed and the proteins which had specifically bound to the column were eluted with 0.5M methyl-alpha-D-mannopyranoside. Most, but not all, of the activity interfering with TNF-alpha binding to its receptor was found to specifically adsorb to the lectin and could be eluted with methyl-alpha-D-mannopyranoside.

20 A sample of 3.5 mg of the Concanavalin-A eluted proteins was dialysed against PBS and fractionated by gel filtration chromatography on a 2 x 45 cm Ultrogel ACA 44 column (LKB, Sweden). Absorption of the eluted proteins, at 258 nm, was determined (—). Fractions of 2 ml were collected and examined at a dilution of 1:20 for their ability to protect against TNF-alpha by following assay procedures 2.1 (x-x) and 2.2 described hereinafter, said last assay being modified so that TNF-alpha was applied at a concentration of 75 U/ml and Balb/c-CL.7 cells were used in the assay. Viability of the cells was examined 12 hours later by 25 determining the uptake of neutral red dye (●—●) (Fig. 1A).

25 An identical sample of the proteins eluting from Concanavalin-A was subjected to 48 hours dialysis against distilled water and then spun to remove insoluble proteins. It was lyophilized and then reconstituted in PBS and subjected to chromatography on the Ultrogel ACA 44 column as above. Fractions were collected and assayed as above. There is no significant change in the fractionation pattern of the protective 30 activity (Fig. 1B). When compared with the retention time of molecular weight markers (bovine serum albumin 67 kDa, ovalbumin 43 kDa, soybean trypsin inhibitor 20.1 kDa and cytochrome c 12.3 kDa), the activity was found to elute somewhat prior to the major protein peak with maximal activity at an apparent molecular weight of about 50 to 70 kDa.

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TABLE I:

5 EXAMINATION OF THE EFFECT OF THE TNF INHIBITORY  
 PROTEIN-CONTAINING URINE CONCENTRATE BY APPLYING  
 IT ON CELLS PRIOR TO, OR TOGETHER WITH TNF-ALPHA

10 Binding of  $^{125}\text{I}$ -TNF-alpha to cells. Effect of the TNF  
 Inhibitory Protein when applied:

15

	<u>A</u>	<u>B</u>	<u>C</u>		
20	Urine donor	At 4°C 90' prior to application of TNF-alpha	At 37°C 90' prior to application of TNF-alpha	At 4°C together with TNF-alpha	
25	none	5290	100%	4630	100%
	1	4600	87%	880	19%
	2	5260	99%	2450	53%
30	3	5160	98%	910	20%
	4	4560	86%	1340	29%

35 Decrease of  $^{125}\text{I}$ -TNF-alpha binding to the cells by the TNF Inhibitory Protein present in the urine concentrate is observed only when  $^{125}\text{I}$ -TNF-alpha and the protein are applied together on cells and not when the protein is first applied on cells and then removed prior to the application of TNF-alpha. This indicates that the interference with TNF-alpha binding to cells is not due to an effect of the TNF Inhibitory Protein on the cells, nor is it due to presence of TNF-alpha itself in the urine, but it rather reflects some kind of interaction between the protein of the invention and TNF-alpha.

40 2. ASSAYS FOR THE TNF INHIBITORY PROTEIN OF THE INVENTION

Two assay procedures were used for monitoring the activity of the TNF Inhibitory Protein in the different fractions during the purification process.

45 2.1 Inhibition of binding of TNF-alpha to its receptor

The assay procedure for the quantitation of TNF binding to cells was performed as described (Israel, S. et al. (1986) Immunol. Letters 12: 217-224; Holtmann, H. and Wallach, D. (1987) J. Immunol. 139: 1161-1167). Cells (HeLa or FS11 foreskin fibroblast cells) were seeded in DMEM (Dulbecco's Modified Eagle's Minimal Essential Medium) at a density of  $2.5 \times 10^5$  cells/well in 15mm well plates. After a 24-hour incubation at 37°C in 5% CO<sub>2</sub>, the plates were transferred to ice, the growth medium was removed and aliquots of the samples containing the TNF Inhibitory Protein were mixed with 10 units of labelled  $^{125}\text{I}$ -TNF-alpha (10<sup>5</sup> cpm) in 0.15 ml phosphate buffered saline (PBS) supplemented with 1 mM Ca<sup>2+</sup> and 1 mM Mg<sup>2+</sup>, 0.5 mg/ml bovine serum albumin (BSA) and 0.1% sodium azide (PBS/BSA) and were applied to the cells and incubated for 2 hours at 4°C. Cells were then rinsed with PBS/BSA, transferred to vials for radioactivity measurement and their associated label was quantitated in a gamma counter. Unspecific binding was determined by adding excess of unlabeled TNF to the assay and the value was subtracted in all the cases.

**2.2 Inhibition of the cytotoxic activity of TNF-alpha**

This bioassay was developed based on the cytotoxic effect of TNF on cycloheximide (CHI)-sensitized cells and its quantitation by the neutral-red uptake method, as described in Wallach, D. (1984) J. Immunol. 132: 2464-2469.

- 5 - Samples to be tested for the presence of the protein were diluted two-fold serially, at 4°C, in DMEM and an equal volume of the same medium containing 40 µg/ml TNF-alpha and 400 µg/ml cycloheximide (CHI) is added thereto.
- 10 - Murine A9 cells were seeded in 96-well flat-bottom microtiter plates ( $1.5 \times 10^4$  cells/well) with 100 µl DMEM-CS (DMEM containing 5% fetal calf serum and 5% calf serum).
- 15 - 100 µl aliquots of the serially diluted protein TNF-alpha-CHI mixtures were applied to each well and the cells were further incubated for 14 hours.
- Viability of the cells was determined by incubation with neutral red for 2 hour, washing away excess dye, extracting the neutral red that was taken up by the cells with Sorenson's citrate buffer-ethanol mixture, and quantitating it colorimetrically at 570 nm with a Microelisa Auto-reader.
- 15 - 1 U/ml of TNF inhibitor activity was defined as the dilution factor giving a statistically significant protection from TNF killing ( $p<0.05$ ).

The bioassay is preferably used in the present invention for monitoring the activity of the protein during purification because it is less laborious and does not involve the use of radiolabelled material. There is no need to transfer the cells from individual wells to counting vials and multiple assays can be scored rather rapidly with the use of the Microelisa Auto-reader.

20 The morphology of murine A9 cells treated under conditions according to this bioassay is shown in Figure 2. In (a) are shown cells incubated with CHI only, in (b) are cells incubated with a TNF-alpha-CHI mixture and in (c) are cells incubated with a TNF-alpha-CHI mixture together with a sample of the TNF Inhibitory Protein (following CM-Sepharose purification, as described hereinafter). The protective effect of the TNF Inhibitory Protein against the cytotoxic effect of TNF-alpha is very clear in (c).

**3. PURIFICATION OF THE TNF INHIBITORY PROTEIN**

In the preferred embodiment of the invention, the substantially purified protein of the invention is produced by a process which comprises:

- 30 a) recovering the crude protein fraction from a dialyzed concentrate of human urine;
- b) subjecting said crude protein fraction of step (a) to ion exchange chromatography to obtain partially purified active fractions of the TNF Inhibitory Protein defined by its ability to inhibit both the binding of TNF to its receptors and the cytotoxic effect of TNF;
- 35 c) applying said partially purified active fractions of the TNF Inhibitory protein from step (b) to reversed phase high pressure liquid chromatography (HPLC) to obtain substantially purified active fractions of the TNF Inhibitory Protein defined by its ability to inhibit both the binding of TNF to its receptors and the cytotoxic effect of TNF; and
- d) recovering the substantially purified protein of step (c) which moves as a single peak on reversed phase HPLC.

40 In one embodiment of said process, the substantially purified protein has a molecular weight of about 26 to 28 kDa on SDS Page under reducing conditions.

45 The ion exchange chromatography of step (b) is preferably performed in 3 steps and includes chromatographic purification in Carboxymethyl Sepharose, Mono S HR 5/5 FPLC and Mono Q HR 5/5 FPLC columns, preferably in this sequence. The reversed phase HPLC is preferably performed in an Aquapore RP300 column.

50 In a further preferred embodiment, in all steps of the purification, the procedure was monitored by measuring the protein concentration (absorbance at 280 nm or relative fluorescence following automatic reaction of representative aliquots with fluorescamine) and the inhibition of the TNF-alpha cytotoxic activity according to the bioassay described in 2.2 above.

**3.1 Preparation of the urine concentrate**

A pool of 200 l male urine from healthy donors was subjected to micro filtration on a Pellicon membrane with a pore size of 0.45 µm. The filtrate was concentrated by ultrafiltration using a Pellicon membrane with a molecular weight cut-off of 10 kDa to a final volume of 500 ml. The concentrate was dialyzed against phosphate buffered saline containing 1 mM benzamidine and 0.1% sodium azide.

**3.2 Carboxy Methyl (CM) Sepharose Chromatography**

55 A 2.7 x 10 cm CM-Sepharose cation exchange column (Pharmacia) was prewashed with 1 M NaCl, 10 mM citric acid buffer, pH 5.0, containing 0.02% sodium azide (buffer C) and equilibrated with 10 mM

5 citric acid buffer, pH 5.0, containing 0.02% sodium azide (buffer A). The urine concentrate of step 3.1 above was dialysed against 2 changes of 100x sample volume buffer A and spun for 15 minutes at 8000 rpm. The supernatant was applied at 4 °C on the CM-Sepharose column at a flow rate of 2 ml/minute and fractions of 50 ml were collected. The column was washed with buffer A until no protein could be detected (about 1500 ml) and then eluted with 5x col. volumes of 200 mM NaCl, 10 mM citric acid buffer, pH 5.0, containing 0.02% sodium azide (buffer B) (5 fractions) followed by elution with 3x col. volumes of buffer C (3 fractions). The fractions were collected and tested as indicated. The major portion of the biological activity of the TNF inhibitory protein was found in the second fraction of the elution with buffer B.

10 3.3 Cation-Exchange Mono S, HR 5/5 FPLC Chromatography

The Mono S HR 5/5 column (Pharmacia) was prewashed with a 10 mM citric acid buffer, pH 5.0, containing 0.02% sodium azide (buffer A) until a stable baseline was demonstrated (monitored at 280 nm by a UV detector). The active fractions eluted from the CM-Sepharose column were pooled and dialyzed against 2 changes of 100x sample volume buffer A. The sample was injected in 8 x 2 ml portions onto the column until the maximum binding capacity of the column was reached (28 mg). The column was washed with buffer A until a flat baseline was seen. The bound proteins were eluted with a linear NaCl gradient (0-350 mM) in buffer A. The gradient was run for 40 minutes at a flow rate of 0.5 ml/minute. Then the column was washed for 10 minutes with 350 mM NaCl in Buffer A (Buffer D.). The proteins which could not be eluted in a concentration of 350 mM NaCl were then eluted from the column with Buffer C. Fractions of 0.5 ml were collected and assayed as indicated. The results are shown in Figure 3. The major portion of the activity was found to elute in fractions 20-23, corresponding to 180-220 mM NaCl.

15 3.4 Anion-exchange Mono Q HR 5/5 FPLC Chromatography

The Mono Q HR 5/5 column (Pharmacia) was prewashed with a 5 mM sodium borate buffer, pH 9.0, containing 0.02% sodium azide (buffer E) until a stable baseline was achieved. The active fractions eluted from the Mono S column were pooled and dialyzed against 2 changes of 100x sample volume buffer E. The sample was injected in 2 ml portions onto the column and the column was run with buffer E until the baseline was flat. The bound proteins were eluted by a 30 mM linear NaCl gradient of 0-60 mM in buffer E, followed by a 30 minute linear gradient from 60 to 300 mM NaCl in buffer E. The column was then washed for 10 minutes with 300 mM NaCl in Buffer E and for 4 minutes with a 1 M NaCl in Buffer E at a flow rate of 0.5 ml/minute. Fractions of 0.5 ml were collected and tested for activity and for protein content. As shown in Figure 4, the majority of the activity eluted in fractions 15-18 at a NaCl concentration of approximately 40 mM.

20 3.5 Reversed-phase high pressure liquid chromatography (HPLC)

The reversed-phase HPLC column Aquapore RP 300 4.6 x 30 mm (Brownlee Labs) was prewashed with 0.3% aqueous trifluoroacetic acid (TFA) (Buffer F) until a stable baseline was obtained by the fluorescamine detection system. The active fractions which were eluted from the Mono Q column were pooled and injected in one 1.6 ml portion onto the column. The column was run with Buffer F at a flow rate of 0.5 ml/minute until the fluorometer did not detect any protein. The column was then eluted at a flow rate of 0.5 ml/minute, with a 0-20% linear gradient of acetonitrile in Buffer F for 5 minutes, followed by a 20-50% linear gradient of acetonitrile for 60 minutes and finally a 50% to 80% linear acetonitrile gradient for 5 minutes. The column was then washed for 15 minutes with 80% acetonitrile. Fractions of 0.5 ml were collected and tested for protein content and for activity. As shown in figure 4, the activity sharply eluted in fractions 21-23 (peaking in fraction 22) together with an isolated protein peak. These fractions corresponded to 27% acetonitrile.

25 3.6 SDS-PAGE

In order to monitor the result of the purification, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed (Figure 6) according to the method of Laemmli U.K., et al. (1970) Nature 227:680. A sample of the active fractions eluting from the ion exchange column of steps 3.2, 3.3 and 3.4 containing 5 µg of protein (Lane B: active fraction eluted from CM-Sepharose column; lane C: active fractions eluted from Mono S column; and lane D: active fractions eluted from Mono Q column) or a sample of 40 µl of the fractions 21-23 (lanes E to G) derived from the reversed phase HPLC, were mixed with 3 x concentrated sample buffer containing 6% SDS (w/v) and 15% v/v mixture of molecular weight markers (alpha-lactalbumin 14.4 kDa, soya bean trypsin inhibitor 20.1 kDa, carbonic anhydrase 30 kDa, ovalbumin 43 kDa, bovine serum albumin 67 KDa, and phosphorylase b. 94 kDa) was treated as above and loaded on lane A. A blank with sample buffer was run on lane H. The gel was run at 160 volt and the protein bands were visualized by silver staining (Oakley, B.R. et al. Anal.

Biochem. 105:361). As shown in figure 6, the purified TNF Inhibitory Protein moved as a single band with an apparent molecular weight of 26-28 kDa (Lanes E-G).

3.7 Automated protein micro-sequence analysis

Samples of the substantially purified TNF Inhibitory Protein of the invention (1-5 µg, 50-200 pmol each) were applied to pretreated, biobrene-coated glass-fiber discs. The dried discs were subjected to repetitive cycles of Edman degradation in an automated pulsed liquid-gas-phase protein microsequencer (Model 475) with an on-line HPLC PTH-amino acid analyzer (Model 120) and a data acquisition and processing unit Model 900, all from Applied Biosystems Inc. Foster City, CA, U.S.A.). The computer-processing unit Model 900, all from Applied Biosystems Inc. Foster City, CA, U.S.A.). The computer-derived sequence was compared with the raw data and was corrected when necessary. Altogether three separate analyses were performed in order to confirm the sequence data. The initial yield was over 40%, indicating that the major protein in the preparation (the 27 kDa band) is related to the resulting sequence. The N-terminal sequencing of the TNF Inhibitory Protein gave the following amino acid sequence:

15	1	5	10	15
	Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser			

The amino acid at the 14th position was not identified. As to the cysteine residue at the 4th position, its presence is theoretical since PTH (Phenyl thiohydantoin) cys cannot be identified as such and no other amino acid residue was detected in this position. A computerized search in the National Biomedical Research Foundation protein library (update No.16) by the FASTP method did not reveal a significant homology to any known protein.

25 4. GENETIC ENGINEERING OF THE TNF INHIBITORY PROTEIN

This invention further concerns DNA molecules comprising the nucleotide sequence coding for the TNF Inhibitory Protein of the invention, replicable expression vehicles containing said DNA molecules, hosts transformed therewith and the TNF Inhibitory Protein produced by expression of such transformed hosts.

30 The term "DNA molecules" includes genomic DNA, cDNA, synthetic DNA and combinations thereof.

30 The cloning of the TNF Inhibitory Protein may be carried out by different techniques. According to one approach, specific antibodies (polyclonal or monoclonal) to the TNF Inhibitory Protein are produced and used to clone the TNF Inhibitory Protein cDNA. This approach is comprised of the following three steps:

35 a) Preparation of antibodies: The antibodies to the TNF Inhibitory Protein can be produced either by using the substantially purified TNF Inhibitory Protein of the present invention or by using one or more synthetic peptides identical to the known sequence of the protein, e.g. the N-terminal protein sequence, or by fusing one of the possible nucleotide sequences deduced from the amino acid sequence of the TNF Inhibitory Protein to the gene coding for Protein A and expressing the fused Protein A - TNF Inhibitory Protein in E. coli.

40 For obtaining polyclonal antibodies, the substantially purified TNF Inhibitory Protein or the synthetic peptides linked to a carrier protein are injected into rabbits. For the production of monoclonal antibodies, the fused Protein A - TNF Inhibitory Protein synthetic gene is expressed in E. coli, the fused protein obtained is purified by affinity chromatography on IgG Sepharose column and injected into mice. Alternatively, the substantially purified TNF Inhibitory Protein of the present invention is injected into mice.

45 b) Screening of TNF Inhibitory Protein producing cells

The antibodies to TNF Inhibitory Protein are used to search for cells producing the TNF Inhibitory Protein by immunofluorescence or by Western blot.

c) Preparation of cDNA from producing cells

50 mRNA is extracted from TNF Inhibitory Protein producing cells and cDNA is prepared by the use of reverse transcriptase. The cDNA is cloned in an expression vector such as λ gt 11 and screened by the use of the antibodies. The λ gt 11 expression vector can be used for insertion of DNA up to 7 kb in length at a unique EcoRI site 53 bases upstream from the beta-galactosidase termination codon. Therefore, foreign sequences DNA may be inserted into this site and expressed under appropriate conditions as fusion proteins. The λgt 11 expression vector is particularly useful for the construction of cDNA libraries to be screened with antibody probes (Huynh, T.V. et al. in:David Glover (ed.), DNA Cloning Techniques: a Practical Approach, IRL Press, Oxford (1984) pp.49-78) as outlined here.

55 Following another approach, a synthetic oligonucleotide or a mixture of synthetic oligonucleotide, whose

sequence is derived from the sequence of a fragment of the protein, e.g. the N-terminal amino acid sequence of the TNF Inhibitory Protein are produced and this oligonucleotides or the mixture of oligonucleotides are used as a probe for cloning the cDNA or the genomic DNA coding for the TNF Inhibitory Protein.

5 The genomic DNA may or may not include naturally occurring introns. It may be obtained, for example, by extraction from suitable cells and purification by means well known in the art. Suitable DNA preparations, such as human genomic DNA, are enzymatically cleaved by restriction enzymes, or randomly sheared, and the fragments inserted into appropriate recombinant vectors to form a gene library. Such vectors can then be screened with synthetic oligonucleotides probes in order to identify a sequence coding for the TNF Inhibitory Protein of the invention.

10 Alternatively, mRNA is isolated from a cell which expresses the protein of the invention and used to produce cDNA by means well known in the art. This cDNA, after conversion to the double-standard form, may be cloned and the resulting clone screened with an appropriate probe for cDNA coding for the desired sequences. Once the desired clone has been isolated, the cDNA may be manipulated in substantially the same manner as the genomic DNA. However, with cDNA there will be no introns or intervening sequences.

15 In order to synthesize the oligonucleotides to be used as probes, it is possible either to perform sequence analysis of the intact TNF Inhibitory Protein or to obtain peptide fragments thereof and to characterize their amino acid sequence. In order to obtain peptide fragments, purified protein preparations are subjected to fragmentation, e.g. by digestion with proteases such as trypsin, chymotrypsin or papain by methods well known in the art (Oike, Y. et al. (1982) J. Biol. Chem. 257: 9751-9758). The peptide fragments produced by digestion are separated by reverse phase HPLC and sequenced by automatic amino acid sequencing techniques.

20 As already described, the sequence corresponding to the first 16 amino acids at the N-terminal portion of the protein was determined by analysis of the substantially purified TNF Inhibitory Protein in an automatic sequencer and the following amino acid sequence was obtained:

1	5	10	15
Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser			

30

Once one or more suitable peptide fragments have been sequenced or a partial sequence of the protein is determined, the DNA sequences capable of encoding them are examined. Due to the degeneration of the genetic code, more than one codon may be used to encode a particular amino acid and one or 35 more different oligonucleotides can be produced, each of which would be capable of encoding the TNF Inhibitory Protein peptide fragments (Watson, J.D., in: Molecular Biology of the Gene, 3rd ed., W.A. Benjamin, Inc. Menlo Park, CA (1977), pp. 356-357). However, only one member of the set contains the nucleotide sequence that is identical to the nucleotide sequence of the gene. Its presence within the set and its capability to hybridize to DNA even in the presence of the other members of the set, makes it possible 40 to employ the unfractionated set of oligonucleotides in the same manner in which one would employ a single oligonucleotide to clone the gene that encodes the peptide. The use of such oligonucleotide or set of oligonucleotides containing the theoretical "most probable" sequence capable of encoding the TNF Inhibitory Protein gene fragments (following the "codon usage rules" disclosed by Lathe, R., et al. (1985) J. Molec. Biol. 183: 1-12) permits to identify the sequence of a complementary oligonucleotide or set of 45 oligonucleotides which is capable of hybridizing to the "most probable" sequence encoding the TNF Inhibitory Protein or at least a portion thereof, or a set of such sequences. This oligonucleotide containing such a complementary sequence may then be synthesized and employed as a probe to identify and isolate the gene of the TNF Inhibitory Protein of the invention (Maniatis, T. et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (1982)).

50 Once a suitable oligonucleotide, or set of oligonucleotides, which is capable of encoding a fragment of the TNF Inhibitory Protein gene (or which is complementary to such an oligonucleotide, or set of oligonucleotides) is identified using the above-described procedure, it is synthesized and hybridized to a DNA or preferably, to a cDNA preparation derived from cells which are capable of expressing the desired gene, preferably after the source of cDNA has been enriched for the desired sequences, e.g. by extracting RNA from cells which produce high levels of the desired gene and then converting it to the corresponding cDNA by employing the enzyme reverse transcriptase.

55 Procedures for hybridization of nucleic acids are common knowledge and are disclosed, for example, in Maniatis, T., Molecular Cloning: A Laboratory Manual, op.cit. and in Haymes, B.T., et al., Nucleic Acid

Hybridization: A Practical Approach, IRL Press, Oxford, England (1985). By hybridization with the above nucleotide or set of oligonucleotides probes, it is possible to identify in a cDNA or genomic library, the DNA sequences capable of such hybridization and they are then analyzed to determine to what extent they contain encoding sequences for the TNF Inhibitory Protein of the invention.

5 By the same or similar techniques it has been possible to successfully clone the genes for several human proteins, such as the tissue-type plasminogen activator (Pennica, D., et al. (1983) *Nature* 301: 214-221).

The DNA molecules coding for the TNF Inhibitory Protein of the invention, obtained by the above described methods, are then inserted into appropriately constructed expression vectors by techniques well known in the art (see Maniatis et al., *op. cit.*). Double stranded cDNA is linked to plasmid vectors by homopolymeric tailing or by restriction linking involving the use of synthetic DNA linkers or blunt-ended ligation techniques. DNA ligases are used to ligate the DNA molecules and undesirable joining is avoided by treatment with alkaline phosphatase.

In order to be capable of expressing a desired protein, an expression vector should comprise also specific nucleotide sequences containing transcriptional and translational regulatory information linked to the DNA coding for the desired protein in such a way as to permit gene expression and production of the protein. First, in order for the gene to be transcribed, it must be preceded by a promoter recognizable by RNA polymerase, to which the polymerase binds and thus initiates the transcription process. There are a variety of such promoters in use, which work with different efficiencies (strong and weak promoters). They 15 are different for prokaryotic and eukaryotic cells.

20 The promoters that can be used in the present invention may be either constitutive, for example, the int promoter of bacteriophage  $\lambda$ , the bla promoter of the beta-lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene of pPR325, etc., or inducible, such as the prokaryotic promoters including the major right and left promoters of bacteriophage  $\lambda$  ( $P_L$  and  $P_R$ ) the trp, recA, lacZ, lacI, ompF and gal promoters of *E. coli*, or the trp-lac hybrid promoter, etc. (Glick, B.R., (1987) 25 *J. Ind. Microbiol.* 1: 277-282).

25 Besides the use of strong promoters to generate large quantities of mRNA, in order to achieve high levels of gene expression in prokaryotic cells, it is necessary to use also ribosome-binding sites to ensure that the mRNA is efficiently translated. One example is the Shine-Dalgarno sequence (SD sequence) 30 appropriately positioned from the initiation codon and complementary to the 3'-terminal sequence of 16S RNA.

For eukaryotic hosts, different transcriptional and translational regulatory sequences may be employed, depending on the nature of the host. They may be derived from viral sources, such as adenovirus, bovine papilloma virus, Simian virus, or the like, where the regulatory signals are associated with a particular gene 35 which has a high level of expression. Examples are the TK promoter of Herpes virus, the SV40 early promoter, the yeast gal4 gene promoter, etc. Transcriptional initiation regulatory signals may be selected which allow for repression and activation, so that expression of the genes can be modulated.

The DNA molecules comprising the nucleotides sequence coding for the TNF Inhibitory Protein of the invention and the operably linked transcriptional and translational regulatory signals is inserted into a vector 40 which is capable of integrating the desired gene sequences into the host cell chromosome. The cells which have stably integrated the introduced DNA into their chromosome can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may provide for prototrophy to an auxotrophic host, biocide resistance, e.g. antibiotics, or heavy metals, such as copper, or the like. The selectable marker gene can either be directly linked to the DNA gene sequences 45 or to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of single chain binding protein mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. cDNA expression vectors incorporating such elements include those described by Okayama, H., (1983) *Mol. Cel. Biol.* 3: 280.

50 In a preferred embodiment, the introduced DNA molecule will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

55 Preferred prokaryotic vectors include plasmids such as those capable of replication in *E. coli*, for example, pBR322, ColE1, pSC101, pACYC 184, etc. (see Maniatis et al., Molecular Cloning: A Laboratory Manual, *op. cit.*); *Bacillus* plasmids such as pC194, pC221, pT127, etc., (Gryczan, T., The Molecular Biology of the Bacilli, Academic Press, NY (1982), pp.307-329); *Streptomyces* plasmids including pIJ101 (Kendall,

K.J., et al., (1987) J. Bacteriol. 169: 4177-4183); Streptomyces bacteriophages such as ØC31 (Chater, K.F. et al., in: Sixth International Symposium on Actinomycetales Biology, Akademiai Kaido, Budapest, Hungary (1986), pp. 45-54), and Pseudomonas plasmids (John, J.F., et al. (1986) Rev. Infect. Dis. 8: 693-704), and Izaki, K. (1978) Jpn. J. Bacteriol. 33: 729-742).

Preferred eukaryotic plasmids include PBV, vaccinia, SV40, 2-micron circle, etc., or their derivatives. Such plasmids are well known in the art (Botstein, D., et al. (1982) Miami Wint. Symp. 19: 265-274; Broach, J.R., in: The Molecular Biology Of the Yeast Saccharomyces: Life Cycle and Inheritance, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 445-470 (1981); Broach, J.R., (1982) Cell 28: 203-204; Bollon, D.P., et al., (1980) J. Clin. Hematol. Oncol. 10: 39-48; Maniatis, T. in: Cell Biology: A Comprehensive Treatise, Vol. 3: Gene Expression, Academic Press, NY, pp. 563-608 (1980)).

Once the vector or DNA sequence containing the construct(s) has been prepared for expression, the DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means: transformation, transfection, conjugation, protoplast fusion, electroporation, calcium phosphate-precipitation, direct microinjection, etc.

Host cells to be used in this invention may be either prokaryotic or eukaryotic. Preferred prokaryotic hosts include bacteria such as E. coli, Bacillus, Streptomyces, Pseudomonas, Salmonella, Serratia, etc. The most preferred prokaryotic host is E. coli. Bacterial hosts of particular interest include E. coli K12 strain 294 (ATCC 31446), E. coli X1776 (ATCC 31537), E. coli W3110 (F<sup>-</sup>, lambda<sup>-</sup>, prototrophic (ATCC 27325)), and other enterobacterium such as Salmonella typhimurium or Serratia marcescens and various Pseudomonas species. Under such conditions, the protein will not be glycosylated. The prokaryotic host must be compatible with the replicon and control sequences in the expression plasmid.

Preferred eukaryotic hosts are mammalian cells, e.g. human, monkey, mouse and chinese hamster ovary (CHO) cells, because they provide post-translational modifications to protein molecules including correct folding or glycosylation at correct sites. Also yeast cells can carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene products and secretes peptides bearing leader sequences (i.e., pre-peptides).

After the introduction of the vector, the host cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene sequences(s) results in the production of the desired TNF Inhibitory Protein or a fragment thereof. The expressed protein is then isolated and purified in accordance with the purification method described in the present application (sector 3 supra) or by any other conventional procedure involving extraction, precipitation, chromatography, electrophoresis, or the like.

A further purification procedure that may be used in preference for purifying the protein of the invention is affinity chromatography. For this purpose, monoclonal antibodies to the TNF Inhibitory Protein are produced and immobilized on a gel matrix contained within a column. Impure preparations containing the recombinant protein are passed through the column. The protein will be bound to the column by the specific antibody while impurities will pass through. After washing, the protein is eluted from the gel by a change in pH or ionic strength.

The monoclonal antibodies used in the present invention can be prepared using conventional hybridoma technique (Kohler et al. (1975) Nature 256: 495; Kohler et al. (1976) Eur. J. Immunol. 6: 511). In general, such procedures involve immunizing an animal with the desired purified protein antigen or with a synthetic peptide having the N-terminal sequence of the desired protein conjugated to a suitable carrier, such as bovine serum albumin. Spleen cells of such animals are isolated and fused with a suitable myeloma cell line. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium and then cloned. The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding the TNF Inhibitory Protein. After identification, the desired clone can be grown in bulk, either in suspension culture or in ascitic fluid by injecting the cells into the peritoneum of suitable host mice.

The monoclonal antibodies produced by said hybridomas, after purification and immobilization, are very efficient for the purification of the TNF Inhibitory Protein in affinity purification procedure using an immuno-adsorbent column.

## 5. UTILITY AND COMPOSITIONS

The TNF Inhibitory Protein, salts, functional derivatives and active fraction thereof and mixtures of any of the foregoing are indicated for antagonizing the deleterious effects of TNF in mammals, i.e. for treating conditions wherein excess of TNF is formed endogenously or is exogenously administered.

The present invention further relates to pharmaceutical compositions comprising a pharmaceutically acceptable carrier and the TNF Inhibitory Protein of the invention or its salts, functional derivatives or active fractions thereof or mixtures of any of the foregoing, as active ingredient(s). These compositions may be used in any condition where there is an over production of endogenous TNF, such as in cases of septic shock, cachexia, graft-versus host reactions, autoimmune diseases like rheumatoid arthritis, etc.. The way of administration can be via any of the accepted modes of administration for similar agents and will depend on the condition to be treated, e.g., intravenously in case of septic shock or local injection in case of rheumatoid arthritis, (for example, into the knee), or continuously by infusion, etc.. The compositions may also be used in cases of TNF intoxication caused by exogenous administration of excessive amounts (overdoses) of TNF.

The pharmaceutical compositions of the invention are prepared for administration by mixing the protein or its derivatives with physiologically acceptable carriers, stabilizers and excipients, and prepared in dosage form, e.g. by lyophilization in dosage vials. The amount of active compound to be administered will depend on the route of administration, the disease to be treated and the condition of the patient. Local injection in case of inflammatory conditions of rheumatoid arthritis will require TNF Inhibitory Protein on a body weight basis than will intravenous infusion in case of septic shock.

### Claims

- 20 1. A Tumor Necrosis Factor (TNF) Inhibitory Protein having the following features:
  - (a) it inhibits the binding of TNF to its receptors and the cytotoxic effect of TNF;
  - (b) when crude urine preparations thereof are chromatographed on an Ultrogel AcA 44 filtration column, the major peak of TNF inhibitory activity elutes slightly before the majority of the protein and shows an apparent molecular weight of about 40-80 KDa; and
  - (c) when crude urine preparations thereof are analysed, the isoelectric point of the active protein is between pH6 and 8
 or a salt, functional derivative or active fraction thereof, said active fraction having the ability to inhibit the binding of TNF to its receptors and the cytotoxic effect of TNF.
- 30 2. The TNF Inhibitory Protein according to claim 1, obtainable from human urine by
  - (a) submitting the fraction, which is non-dialyzable through a membrane with molecular weight cut-off at 10 KDa to lectin affinity chromatography;
  - (b) eluting the protein adsorbed to the lectin;
  - (c) subjecting the eluted protein to gel filtration or to ion exchange chromatography; and
  - (d) recovering those fractions which inhibit the binding of TNF to its receptors and the cytotoxic effect of TNF.
- 35 3. The TNF Inhibitory Protein of claim 1 or 2 in substantially purified form.
- 40 4. The TNF Inhibitory Protein of any one of claims 1 to 3 having a molecular weight of about 26-28 KDa when the substantially purified protein is analyzed by SDS PAGE under reducing conditions.
- 45 5. The TNF Inhibitory Protein of any one of claims 1 to 4 moving as a single peak on reversed phase high performance liquid chromatography (HPLC).
6. The TNF Inhibitory Protein of any one of claims 1 to 5 having the ability to inhibit the binding of TNF- $\alpha$  to its cell surface receptors on human HeLa and FS11 fibroblast cells.
- 50 7. The TNF Inhibitory Protein of any one of claims 1 to 6 having the ability to inhibit the cytotoxic effect of TNF- $\alpha$  on murine A9 cells.
- 55 8. The TNF Inhibitory Protein of any one of claims 1 to 7 which contains the following amino acid sequence at its N-terminal portion

1                    5                    10  
**Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-**  
15  
5                    **Gln-X-Asn-Ser**

wherein X is an unidentified amino acid residue.

- 10 9. The TNF Inhibitory Protein according to any one of claims 1 to 8 which is isolated from urine.
- 10. The TNF Inhibitory Protein according to claim 9 wherein the urine is human urine.
- 11. A process for the isolation of substantially purified TNF Inhibitory Protein which comprises:
  - (a) recovering the crude protein fraction from a dialyzed concentrate of human urine;
  - (b) subjecting said crude protein fraction of step (a) to ion exchange chromatography to obtain partially purified active fractions of the TNF Inhibitory Protein defined by its ability to inhibit both the binding of TNF to its receptors and the cytotoxic effect of TNF;
  - (c) applying said partially purified active fractions of the TNF Inhibitory Protein from step (b) to reversed phase HPLC to obtain substantially purified active fractions of the TNF Inhibitory Protein defined by its ability to inhibit both the binding of TNF to its receptors and the cytotoxic effect of TNF; and
  - (d) recovering the substantially purified protein of step (c) which moves as a single peak on reversed phase HPLC.
- 12. The process according to claim 11 wherein said substantially purified protein has a molecular weight of about 26-28 KDa on SDS PAGE under reducing conditions.
- 13. The process according to claim 11 or 12 wherein the ion exchange chromatography of step (b) is performed in 3 steps and includes chromatographic purification in Carboxymethyl Sepharose, Mono S HR 5/5 FPLC and Mono Q HR 5/5 FPLC columns, preferably in this sequence.
- 14. The process according to any one of claims 11 to 13 wherein the activity of the fractions in steps (b), (c) and (d) is defined by the ability of the TNF Inhibitory Protein to inhibit the binding of TNF- $\alpha$  to its cell surface receptors on human HeLa and FS11 fibroblast cells.
- 15. The process according to any one of claims 11 to 14 wherein the activity of the fractions in steps (b), (c) and (d) is defined by the ability of the TNF Inhibitory Protein to inhibit the cytotoxic effect of TNF- $\alpha$  on murine A9 cells.
- 16. The TNF Inhibitory Protein according to any one of claims 1 to 10 produced by the process of any one of claims 11 to 15.
- 17. The TNF Inhibitory Protein of any one of claims 1 to 8 which is a recombinant protein.
- 18. The TNF Inhibitory Protein of claim 17 which is produced in a prokaryotic host, preferably in E. coli, or in a eukaryotic host, preferably in a mammalian cell.
- 19. A DNA molecule comprising a nucleotide sequence coding for the TNF Inhibitory Protein or a functional derivative or active fraction thereof of any one of claims 1 to 10, wherein said TNF Inhibitory Protein contains the following amino acid sequence at its N-terminal portion

1                    5                    10  
**Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-**  
15  
55                    **Gln-X-Asn-Ser**

wherein X is an unidentified amino acid residue and wherein said active fraction has the ability to inhibit the binding of TNF to its receptors and the cytotoxic effect of TNF.

20. The DNA molecule of claim 19 wherein the nucleotide sequence is a genomic DNA sequence or a  
5 cDNA sequence.
21. A replicable expression vehicle comprising the DNA molecule of claim 19 or 20 and capable, in a  
transformant host cell, of expressing a TNF Inhibitory Protein according to any one of claims 1 to 10,  
17 or 18.
- 10 22. A host cell transformed with the replicable expression vehicle of claim 21.
23. The host cell according to claim 22 which is a prokaryotic host cell.
- 15 24. The host cell according to claim 22 which is a eukaryotic host cell.
25. A process for producing a TNF Inhibitory Protein comprising the steps of  
(a) culturing a transformed host cell according to any one of claims 22 to 24 in a suitable culture  
medium; and  
20 (b) isolating said TNF Inhibitory Protein.
26. A recombinant TNF Inhibitory Protein produced by the process of claim 25.
27. A pharmaceutical composition comprising a TNF Inhibitory Protein according to any one of claims 1 to  
10, 16 to 18 or 26, a salt, functional derivative or active fraction thereof or a mixture of any of the  
25 foregoing as active ingredient(s) together with a pharmaceutically acceptable carrier, wherein said  
active fraction has the ability to inhibit the binding of TNF to its receptors and the cytotoxic effect of  
TNF.
- 30 28. The pharmaceutical composition according to claim 27 for antagonizing the deleterious effect of TNF in  
mammals.
29. The pharmaceutical composition according to claim 27 for the treatment of conditions wherein excess  
of TNF is formed endogenously or is exogenously administered.
- 35 30. The use of a protein according to any one of claims 1 to 10, 16 to 18 or 26 for the manufacture of a  
pharmaceutical composition according to any one of claims 27 to 29.
31. An antibody to the TNF Inhibitory Protein according to any one of claims 1 to 10, 16 to 18 or 26.
- 40 32. The antibody according to claim 31 which is a monoclonal antibody.
33. The antibody according to claim 31 or 32 for the purification of the TNF Inhibitory Protein according to  
any one of claims 1 to 10, 16 to 18 or 26.

45 **Patentansprüche**

1. Tumornekrosefaktor (TNF)-inhibierendes Protein mit den folgenden Merkmalen:  
50 (a) es inhibiert die Bindung von TNF an seine Rezeptoren und den cytotoxischen Effekt von TNF;  
(b) wenn Urinrohrpräparationen davon über eine Ultragel AcA 44-Filtrationssäule chromatographiert  
werden, eluiert der Hauptpeak mit TNF-inhibierender Aktivität kurz vor der Hauptmenge an Protein  
und zeigt ein relatives Molekulargewicht von etwa 40 bis 80 kD; und  
(c) wenn Urinrohrpräparationen davon analysiert werden, liegt der isoelektrische Punkt des aktiven  
Proteins zwischen pH 6 und 8;
- 55 oder ein Salz, funktionelles Derivat oder ein aktiver Bestandteil davon, wobei der aktive Bestandteil die  
Bindung von TNF an seine Rezeptoren und den cytotoxischen Effekt von TNF inhibieren kann.
2. TNF-inhibierendes Protein nach Anspruch 1, erhältlich aus menschlichem Urin durch

- (a) Lectinaffinitätschromatographie mit der Fraktion, die nicht durch eine Membran mit einem molekularen Ausschlußvolumen von 10 kD dialysierbar ist;  
(b) Elution des an das Lectin adsorbierten Proteins;  
(c) Gelfiltration oder Ionenaustauschchromatographie mit dem eluierten Protein; und  
(d) Gewinnung derjenigen Fraktionen, die die Bindung von TNF an seine Rezeptoren und den cytotoxischen Effekt von TNF inhibieren.

- 5
3. TNF-inhibierendes Protein nach Anspruch 1 oder 2 in im wesentlichen aufgereinigter Form.  
10 4. TNF-inhibierendes Protein nach einem der Ansprüche 1 bis 3 mit einem Molekulargewicht von etwa 26 bis 28 kD, wenn das im wesentlichen aufgereinigte Protein durch SDS-PAGE unter reduzierenden Bedingungen analysiert wird.  
15 5. TNF-inhibierendes Protein nach einem der Ansprüche 1 bis 4, das als einzelner Peak auf Umkehrphasen-Hochleistungsflüssigkeitschromatographie (HPLC) wandert.  
6. TNF-inhibierendes Protein nach einem der Ansprüche 1 bis 5, das die Bindung von TNF- $\alpha$  an seine Zelloberflächenrezeptoren auf menschlichen HeLa- und FS11-Fibroblasten-Zellen inhibieren kann.  
20 7. TNF-inhibierendes Protein nach einem der Ansprche 1 bis 6, das den cytotoxischen Effekt von TNF- $\alpha$  auf Maus-A9-Zellen inhibieren kann.  
8. TNF-inhibierendes Protein nach einem der Ansprüche 1 bis 7, das die folgende Aminosäuresequenz in  
25 seinem N-terminalen Bereich enthält:

1 5 10  
Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-  
30 15  
Gln-X-Asn-Ser

- 35 worin X ein nicht-identifizierter Aminosäurerest ist.  
9. TNF-inhibierendes Protein nach einem der Ansprüche 1 bis 8, das aus Urin isoliert wird.  
10. TNF-inhibierendes Protein nach Anspruch 9, wobei der Urin menschlicher Urin ist.  
40 11. Verfahren zur Isolierung von im wesentlichen aufgereinigtem TNF-inhibierendem Protein, das umfaßt:  
(a) Gewinnung der Proteinrohfraktion aus einem dialysierten Konzentrat aus menschlichem Urin;  
(b) Ionenaustauschchromatographie mit der Proteinrohfraktion aus Schritt (a), wodurch partiell aufgereinigte aktive Fraktionen des TNF-inhibierenden Proteins erhalten werden, das durch seine Fähigkeit definiert wird, sowohl die Bindung von TNF an seine Rezeptoren als auch den cytotoxi-  
45 schen Effekt von TNF zu inhibieren;  
(c) Umkehrphasen-HPLC mit den partiell aufgereinigten aktiven Fraktionen des TNF-inhibierenden Proteins aus Schritt (b), wodurch im wesentlichen aufgereinigte aktive Fraktionen des TNF-inhibie-  
50 renden Proteins erhalten werden, das durch seine Fähigkeit definiert wird, sowohl die Bindung von TNF an seine Rezeptoren als auch den cytotoxischen Effekt von TNF zu inhibieren; und  
(d) Gewinnung des im wesentlichen aufgereinigten Proteins aus Schritt (c), das als einzelner Peak auf Umkehrphasen-HPLC wandert.  
12. Verfahren nach Anspruch 11, wobei das im wesentlichen aufgereinigte Protein ein Molekulargewicht von etwa 26 bis 28 kD in einer SDS-PAGE unter reduzierenden Bedingungen aufweist.  
55 13. Verfahren nach Anspruch 11 oder 12, wobei die Ionenaustauschchromatographie in Schritt (b) in drei Schritten durchgeführt wird und chromatographische Reinigung in Carboxymethyl-Sepharose-, Mono S

HR 5/5 FPLC- und Mono Q HR 5/5 FPLC-Säulen, vorzugsweise in dieser Reihenfolge, einschließt.

- 5 14. Verfahren nach einem der Ansprüche 11 bis 13, wobei die Aktivität der Fraktionen in den Schritten (b),  
an seine Zelloberflächenrezeptoren auf menschlichen HeLa- und FS11-Fibroblasten-Zellen zu inhibie-  
ren.

10 15. Verfahren nach einem der Ansprüche 11 bis 14, wobei die Aktivität der Fraktionen in den Schritten (b),  
(c) und (d) durch die Fähigkeit des TNF-inhibierenden Proteins definiert wird, den cytotoxischen Effekt  
von TNF- $\alpha$  auf Maus-A9-Zellen zu inhibieren.

15 16. TNF-inhibierendes Protein nach einem der Ansprüche 1 bis 10, hergestellt nach dem Verfahren nach  
einem der Ansprüche 11 bis 15.

20 17. TNF-inhibierendes Protein nach einem der Ansprüche 1 bis 8, das ein rekombinantes Protein ist.

18. TNF-inhibierendes Protein nach Anspruch 17, das in einem prokaryontischen Wirt, vorzugsweise in E.  
coli, oder in einem eukaryontischen Wirt, vorzugsweise in einer Säugerzelle, hergestellt wird.

19. DNA-Molekül, umfassend eine Nucleotidsequenz, die das TNF-inhibierende Protein oder ein funktionel-  
les Derivat oder einen aktiven Bestandteil davon nach einem der Ansprüche 1 bis 10 codiert, wobei das  
TNF-inhibierende Protein die folgende Aminosäuresequenz in seinem N-terminalen Bereich enthält:

1 5 10  
Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-  
15  
Gln-X-Asn-Ser

worin X ein nicht-identifizierter Aminosäurerest ist, und wobei der aktive Bestandteil die Bindung von TNF an seine Rezeptoren und den cytotoxischen Effekt von TNF inhibieren kann.

- 35 20. DNA-Molekl nach Anspruch 19, wobei die Nucleotidsequenz eine genomische DNA-Sequenz oder  
eine cDNA-Sequenz ist.

40 21. Replizierbares Expressionsvehikel umfassend die DNA-Sequenz nach Anspruch 19 oder 20, das TNF-  
inhibierendes Protein nach einem der Ansprche 1 bis 10, 17 und 18 in einer transformierten Wirtszelle  
exprimieren kann.

22. Wirtszelle, die mit dem replizierbaren Expressionsvehikel nach Anspruch 21 transformiert ist.

23. Wirtszelle nach Anspruch 22, die eine prokaryontische Wirtszelle ist.

45 24. Wirtszelle nach Anspruch 22, die eine eukaryontische Wirtszelle ist.

25. Verfahren zur Herstellung eines TNF-inhibierenden Proteins, das die folgenden Schritte umfat:  
     (a) Zchtung einer transformierten Wirtszelle nach einem der Ansprche 22 bis 24 in einem  
     geeigneten Kulturmedium; und  
     (b) Isolierung des TNF-inhibierenden Proteins.

50 26. Rekombinantes TNF-inhibierendes Protein, hergestellt nach dem Verfahren nach Anspruch 25.

55 27. Arzneimittel, enthaltend ein TNF-inhibierendes Protein nach einem der Ansprche 1 bis 10, 16 bis 18  
und 26, ein Salz, funktionales Derivat oder einen aktiven Bestandteil davon, oder ein Gemisch de-  
vorstehend genannten Stoffe als Wirkstoff(e) zusammen mit einem pharmazeutisch vertrglichenen  
Trger, wobei der aktive Bestandteil die Bindung von TNF an seine Rezeptoren und den cytotoxischen

Effekt von TNF inhibieren kann.

28. Arzneimittel nach Anspruch 27 zur Bekämpfung der nachteiligen Wirkungen von TNF in Säugern.
- 5    29. Arzneimittel nach Anspruch 27 zur Behandlung von Zuständen, bei denen ein Überschuß an TNF endogen gebildet oder exogen zugeführt wird.
- 10    30. Verwendung eines Proteins nach einem der Ansprüche 1 bis 10, 16 bis 18 und 26 zur Herstellung eines Arzneimittels nach einem der Ansprüche 27 bis 29.
- 15    31. Antikörper gegen das TNF-inhibierende Protein nach einem der Ansprüche 1 bis 10, 16 bis 18 und 26.
32. Antikörper nach Anspruch 31, der ein monoklonaler Antikörper ist.
- 15    33. Antikörper nach Anspruch 31 oder 32 zur Aufreinigung des TNF-inhibierenden Proteins nach einem der Ansprüche 1 bis 10, 16 bis 18 und 26.

#### Revendications

- 20    1. Protéine inhibitrice du facteur de nécrose des tumeurs (Tumor Necrosis Factor, TNF), présentant les caractéristiques suivantes :
- 25    (a) elle inhibe la fixation du TNF à ses récepteurs, ainsi que l'effet cytotoxique du TNF ;  
       (b) quand on en chromatographie, sur une colonne de filtration sur Ultrogel AcA 44, des préparations d'urine brute, le pic principal de l'activité inhibitrice du TNF subit une élution un peu avant la plus grande partie de la protéine, et présente une masse moléculaire apparente d'environ 40 à 80 kDa, et  
       (c) quand on en analyse une préparation d'urine brute, le point isoélectrique de la protéine active est compris entre pH 6 et 8,  
       ou l'un de ses sels, dérivés fonctionnels ou fractions actives, les fractions actives étant à même d'inhiber la fixation du TNF à ses récepteurs et l'effet cytotoxique du TNF.
- 30    2. Protéine inhibitrice du TNF selon la revendication 1, que l'on peut obtenir à partir de l'urine humaine par les étapes consistant :
- 35    (a) à soumettre à une chromatographie d'affinité de lectine la fraction non dialysable à travers une membrane ayant une masse moléculaire de coupure de 10 kDa ;  
       (b) à éluer la protéine adsorbée par la lectine ;  
       (c) à soumettre la protéine élue à une filtration sur gel ou à une chromatographie par échange d'ions ; et  
       (d) à récupérer les fractions qui inhibent la fixation du TNF à ses récepteurs et l'effet cytotoxique du TNF.
- 40    3. Protéine inhibitrice du TNF selon les revendications 1 ou 2, sous forme essentiellement purifiée.
- 45    4. Protéine inhibitrice du TNF selon l'une quelconque des revendications 1 à 3, ayant une masse moléculaire d'environ 26 à 28 kDa quand la protéine essentiellement purifiée est soumise à une analyse par SDS-PAGE dans des conditions réductrices.
- 50    5. Protéine inhibitrice du TNF selon l'une quelconque des revendications 1 à 4, qui migre sous forme d'un pic unique dans le cadre d'une chromatographie en phase liquide hautes performances en phase inversée (CLHP).
- 55    6. Protéine inhibitrice du TNF selon l'une quelconque des revendications 1 à 5, ayant la possibilité d'inhiber la fixation du TNF- $\alpha$  à ses récepteurs en surface cellulaire sur des cellules de fibroblastes HeLa et FS11 humaines.
- 55    7. Protéine inhibitrice du TNF selon l'une quelconque des revendications 1 à 6, ayant la possibilité d'inhiber l'effet cytotoxique du TNF- $\alpha$  sur les cellules A9 de souris.

8. Protéine inhibitrice du TNF selon l'une quelconque des revendications 1 à 7, qui contient la séquence suivante d'acides aminés sur sa position N-terminale

1                   5                   10  
 Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-  
 5                   15  
 Gln-X-Asn-Ser

10

où X est un résidu d'acide aminé non identifié.

9. Protéine inhibitrice du TNF selon l'une quelconque des revendications 1 à 8, qui est isolée de l'urine.

- 15 10. Protéine inhibitrice du TNF selon la revendication 9, dans laquelle l'urine est l'urine humaine.

11. Procédé pour isoler une protéine inhibitrice du TNF essentiellement purifiée, qui comprend les étapes consistant :

- 20 (a) à récupérer la fraction de protéine brute d'un concentré dialysé d'urine humaine ;  
 (b) à soumettre cette fraction de protéine brute de l'étape (a) à une chromatographie d'échange d'ions pour obtenir des fractions actives partiellement purifiées de la protéine inhibitrice du TNF, définie par son aptitude à inhiber tant la fixation du TNF à ses récepteurs que l'effet cytotoxique du TNF ;  
 25 (c) à soumettre à une CLHP en phase inversée les fractions actives partiellement purifiées de la protéine inhibitrice du TNF de l'étape (b), pour obtenir des fractions actives essentiellement purifiées de la protéine inhibitrice du TNF, définie par son aptitude à inhiber tant la fixation du TNF à ses récepteurs que l'effet cytotoxique du TNF ; et  
 (d) à récupérer la protéine essentiellement purifiée de l'étape (c), qui migre sous forme d'un pic unique en CLHP en phase inversée.

- 30 12. Procédé selon la revendication 11, dans lequel la protéine essentiellement purifiée a une masse moléculaire d'environ 26 à 28 kDa en SDS-PAGE dans des conditions réductrices.

- 35 13. Procédé selon la revendication 11 ou 12, dans lequel la chromatographie d'échange d'ions de l'étape (b) est mise en oeuvre en 3 étapes et comprend une purification chromatographique sur des colonnes de carboxyméthyl Sépharose, de Mono S HR 5/5 FPLC et de Mono Q HR 5/5 FPLC, de préférence dans cet ordre.

- 40 14. Procédé selon l'une quelconque des revendications 11 à 13, dans lequel l'activité des fractions des étapes (b), (c) et (d) est définie par l'aptitude de la protéine inhibitrice du TNF à inhiber la fixation du TNF- $\alpha$  sur ses récepteurs en surface cellulaire, sur des cellules de fibroblaste HeLa et FS11 humaines.

- 45 15. Procédé selon l'une quelconque des revendications 11 à 14, dans lequel l'activité des fractions des étapes (b), (c) et (d) est définie par l'aptitude de la protéine inhibitrice du TNF à inhiber l'effet cytotoxique du TNF- $\alpha$  sur les cellules A9 de souris.

- 50 16. Protéine inhibitrice du TNF selon l'une quelconque des revendications 1 à 10, produite par le procédé selon l'une quelconque des revendications 11 à 15.

17. Protéine inhibitrice du TNF selon l'une quelconque des revendications 1 à 8, qui est une protéine recombinante.

- 55 18. Protéine inhibitrice du TNF selon la revendication 17, qui est produite dans un hôte procaryote, de préférence dans E. coli, ou dans un hôte eucaryote, de préférence dans une cellule de mammifère.

19. Molécule d'ADN comprenant une séquence nucléotidique codant pour la protéine inhibitrice du TNF ou l'un de ses dérivés fonctionnels ou fractions actives, selon l'une quelconque des revendications 1 à 10,

dans laquelle la protéine inhibitrice du TNF contient la séquence suivante d'acides aminés dans sa portion N-terminale

1 5 10  
Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-  
15  
Gln-X-Asn-Ser

10 où X est un résidu d'acides aminés non identifié, et où la fraction active a la possibilité d'inhiber la  
fixation du TNF à ses récepteurs et l'effet cytotoxique du TNF.

- 15 20. Molécule d'ADN selon la revendication 19, dans laquelle la séquence nucléotidique est une séquence  
d'ADN de génome ou une séquence d'ADNc.

20 21. Véhicule d'expression répliable comprenant la molécule d'ADN selon les revendications 19 ou 20 et  
capable, dans une cellule hôte transformante, d'exprimer une protéine inhibitrice du TNF selon l'une  
quelconque des revendications 1 à 10, 17 ou 18.

25 22. Cellule hôte transformée à l'aide du véhicule d'expression répliable de la revendication 21.

25 23. Cellule hôte selon la revendication 22, qui est une cellule hôte procaryote.

30 24. Cellule hôte selon la revendication 22, qui est une cellule hôte eucaryote.

35 25. Procédé pour produire une protéine inhibitrice du TNF, qui comprend les étapes consistant :  
(a) à cultiver une cellule hôte transformée selon l'une quelconque des revendications 22 à 24 dans  
un milieu de culture approprié ; et  
(b) à isoler la protéine inhibitrice du TNF.

40 26. Protéine inhibitrice du TNF recombinante produite par le procédé de la revendication 25.

35 27. Composition pharmaceutique comprenant une protéine inhibitrice du TNF selon l'une quelconque des  
revendications 1 à 10, 16 à 18 ou 26, sel, dérivé fonctionnel ou fraction active de cette dernière, ou  
mélange de l'un quelconque d'entre eux servant de principe ou de principes actifs, avec un excipient  
pharmaceutiquement acceptable, la fraction active pouvant inhiber la fixation du TNF à ses récepteurs  
et l'effet cytotoxique du TNF.

45 28. Composition pharmaceutique selon la revendication 27, destinée à antagoniser l'effet gênant du TNF  
chez les mammifères.

45 29. Composition pharmaceutique selon la revendication 27 pour le traitement d'états dans lesquels il y a  
formation endogène ou administration exogène d'un excès de TNF.

50 30. Utilisation d'une protéine selon l'une quelconque des revendications 1 à 10, 16 à 18 ou 26, pour  
fabriquer une composition pharmaceutique selon l'une quelconque des revendications 27 à 29.

50 31. Anticorps contre la protéine inhibitrice du TNF selon l'une quelconque des revendications 1 à 10, 16 à  
18 ou 26.

55 32. Anticorps selon la revendication 31, qui est un anticorps monoclonal.

55 33. Anticorps selon la revendication 31 ou 32, destiné à purifier la protéine inhibitrice du TNF selon l'une  
quelconque des revendications 1 à 10, 16 à 18 ou 26.

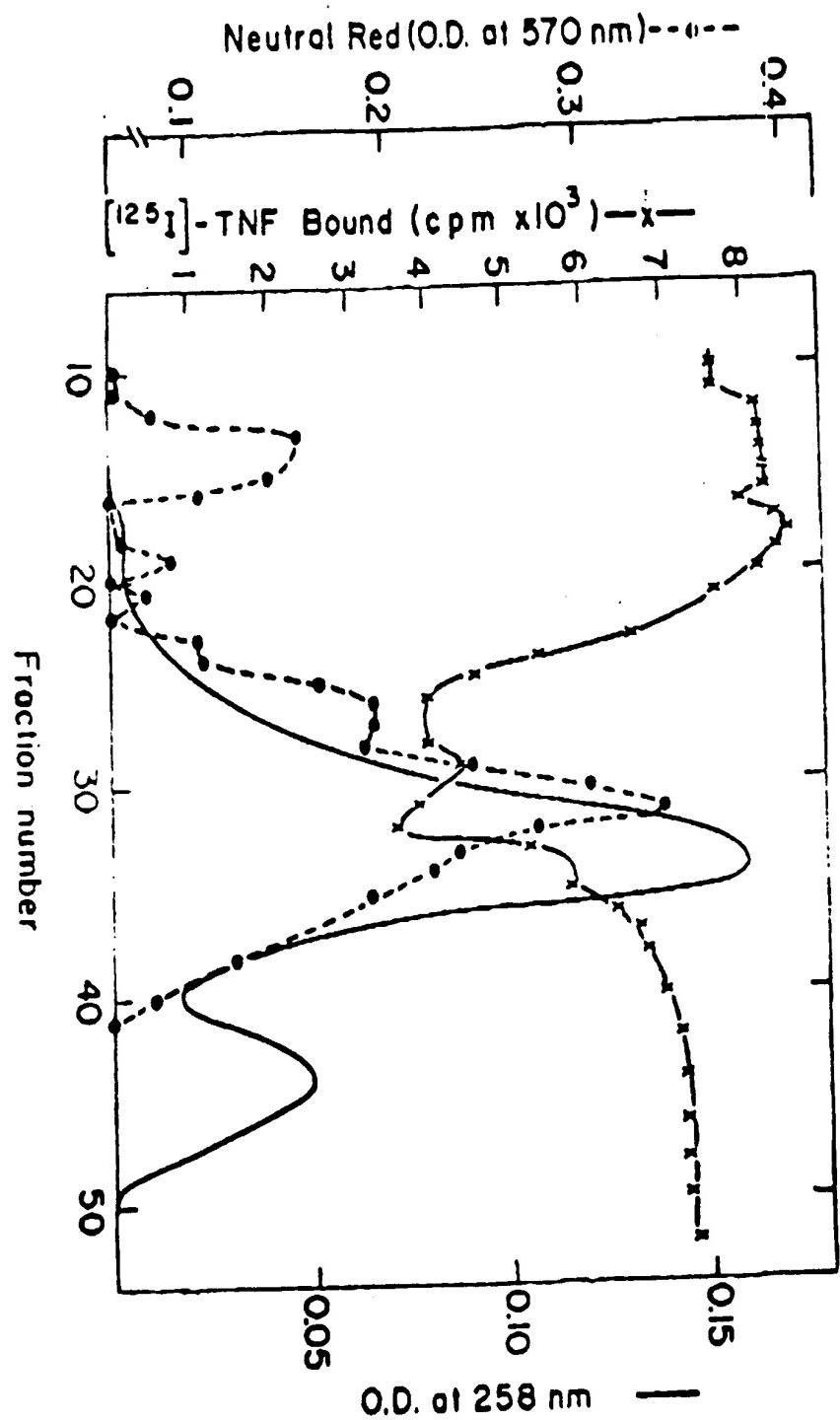


Fig. 1A

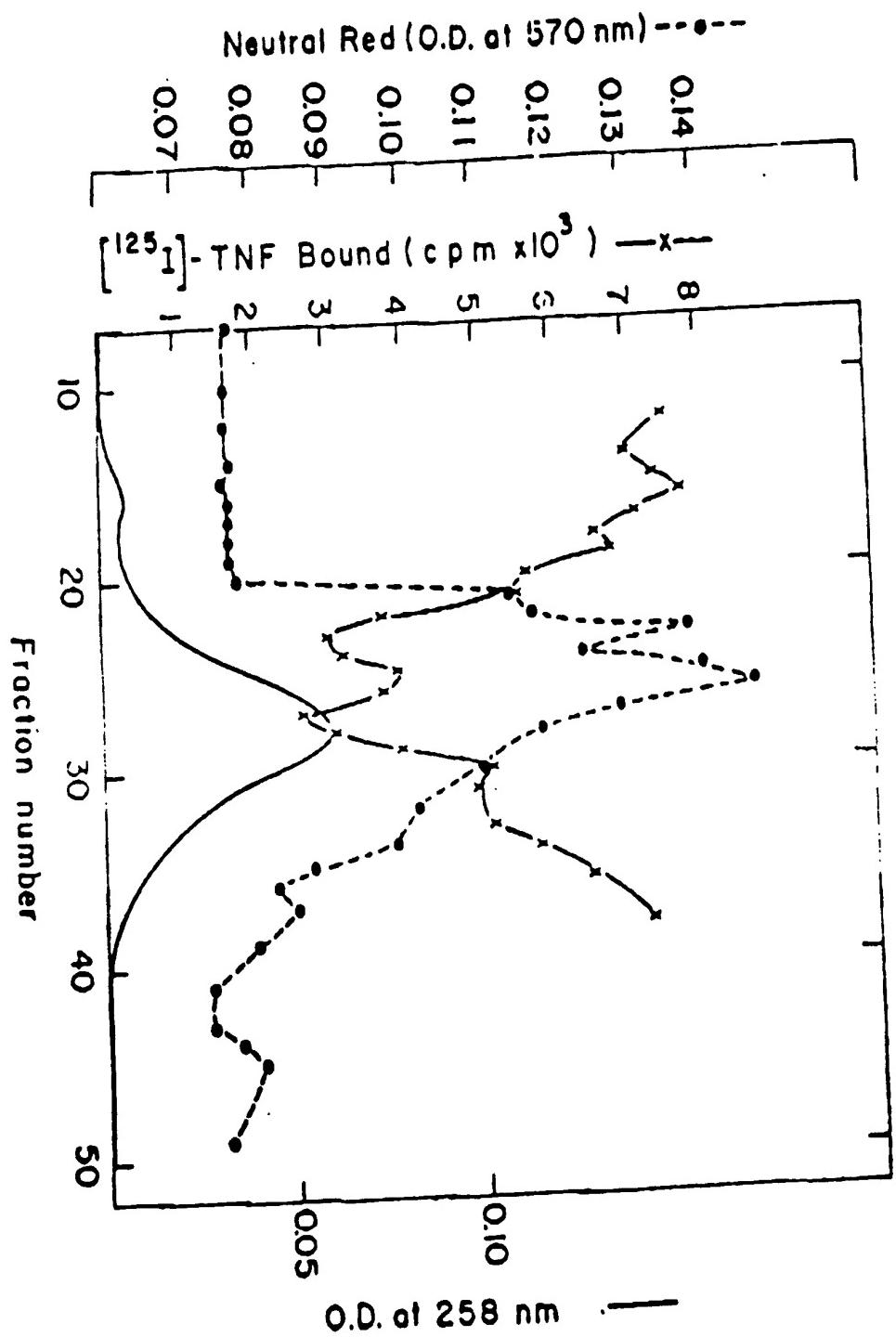
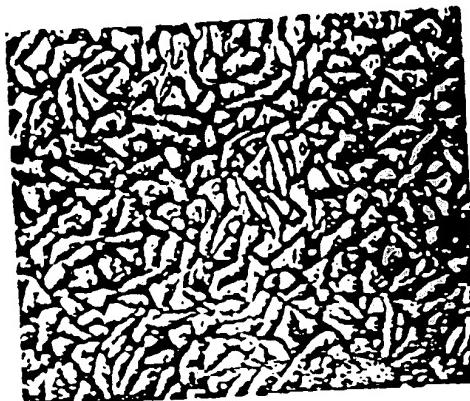
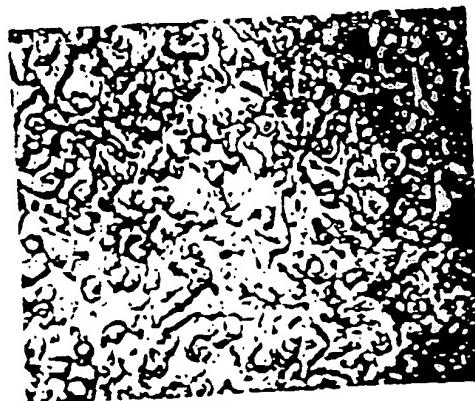


Fig. 1B

(a)



(b)



(c)



Fig. 2

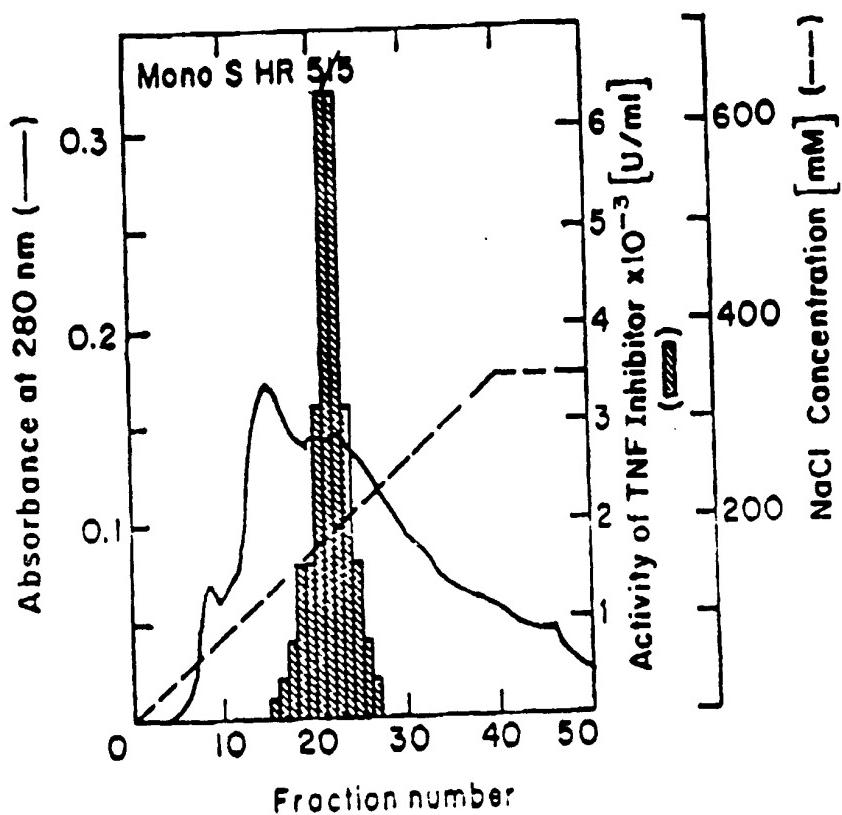


Fig. 3

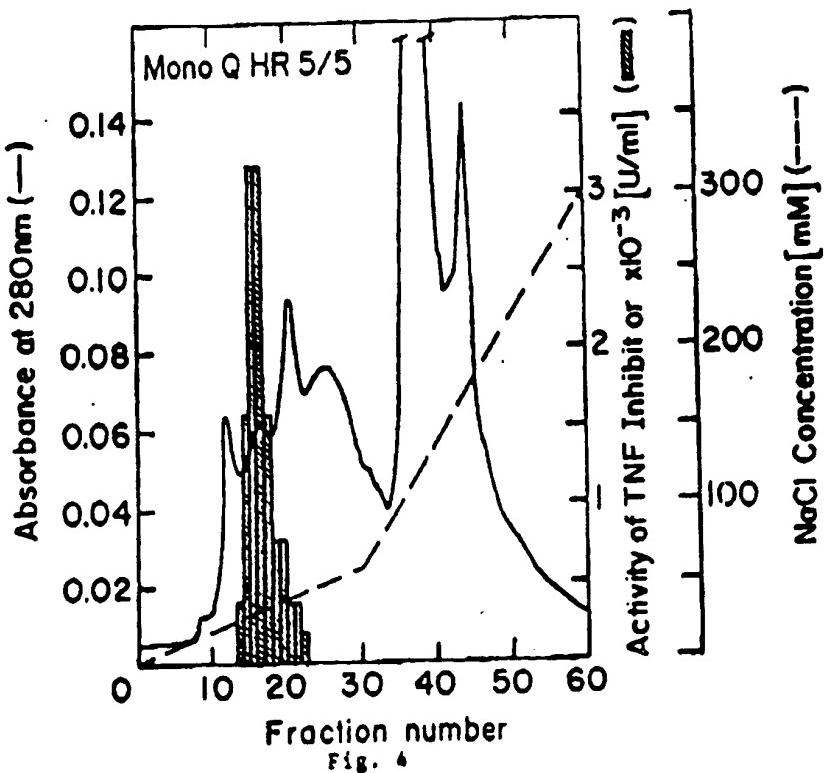


Fig. 4

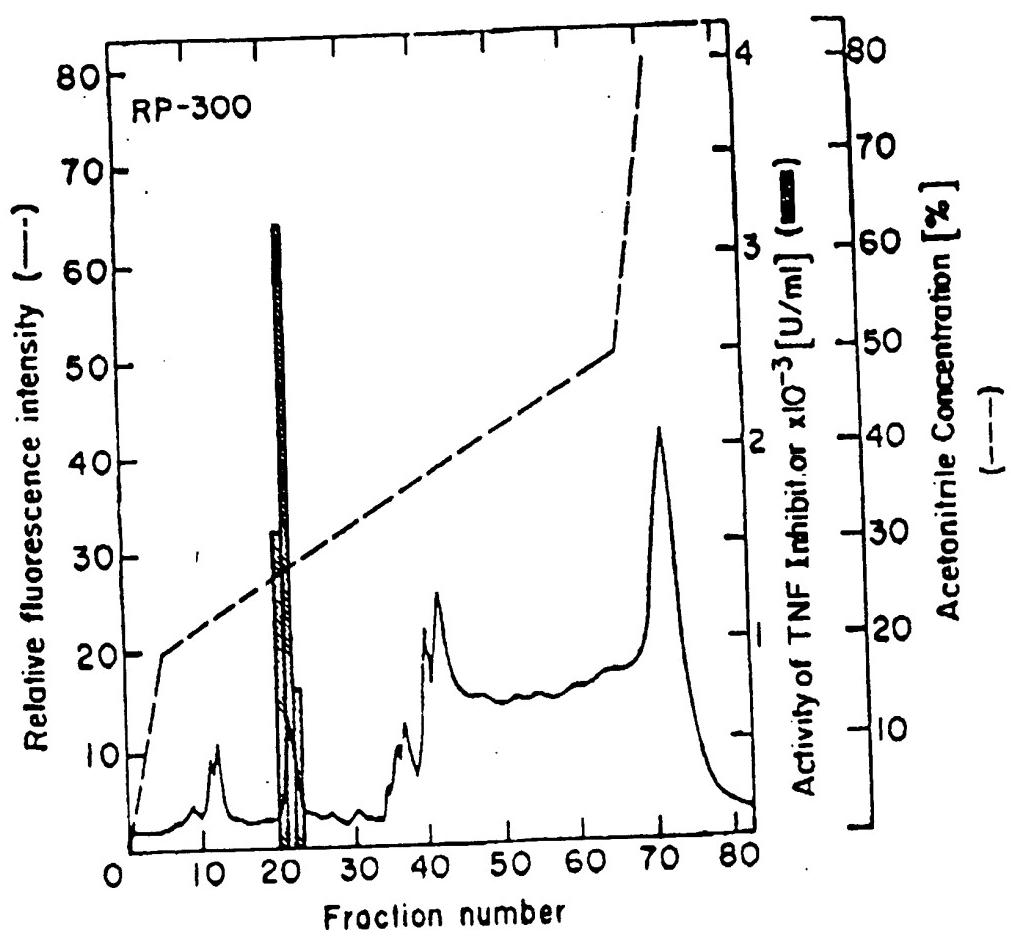


Fig. 5

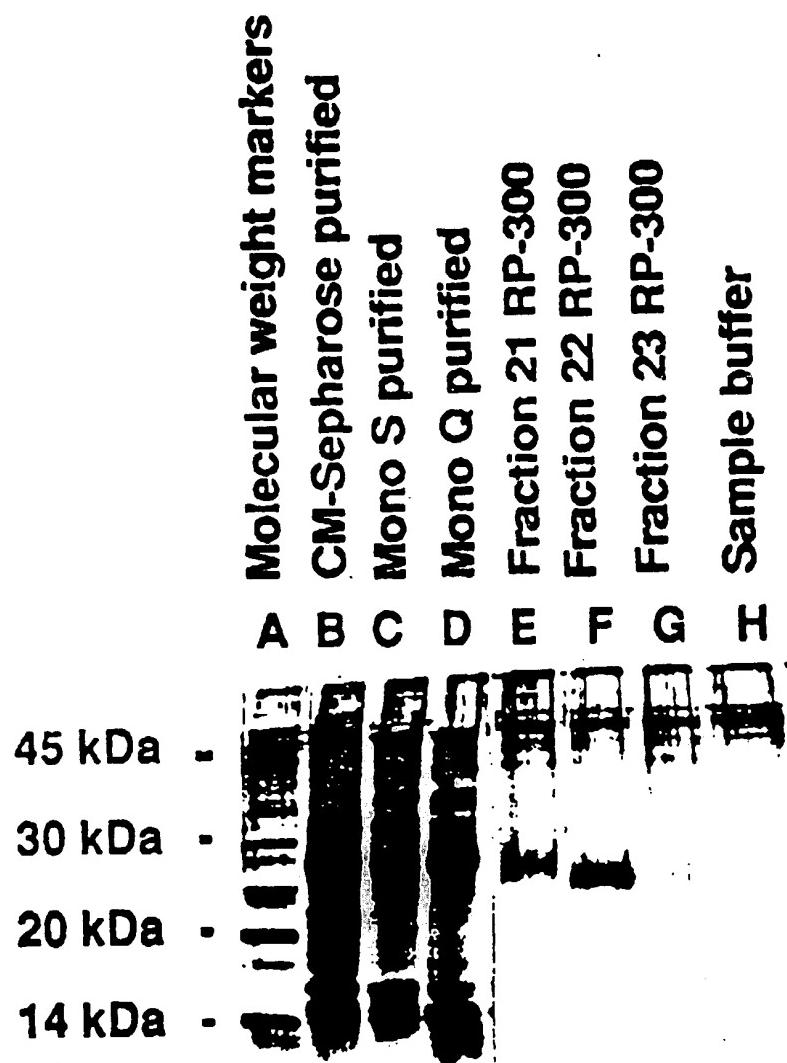


Fig. 6